SUMMARY

This report outlines the progress so far in our statististical population studies which are designed to provide a firm basis for two relatively new forms of sports drug testing; namely the use of carbon isotope ratio mass spectrometry (CIRMS) for detecting the abuse of endogenous anabolic steroids, and the use of isoelectric focussing to detect the abuse of erythropoietin (EPO). The techniques are very different in methodology and application but both rely on detecting small but significant differences between truly endogenous compounds and their synthetic variants. In order to reliably detect doping with either technique it is essential that the extent of natural variation in the elite athlete population be known. Criteria have been set which are already being used to detect doping but so far no large-scale study has been carried out to test these criteria over a wide range of elite athletes. Our study will provide this information which will not only support the validity of the tests but may also result in improvements in the criteria leading to a more robust and sensitive testing program.

The progress so far is to schedule with over 70% of the samples being analysed for ketosteroids for part 1 of the study (CIR measurements) and almost 70% of the samples being completed for part 2 of the study (EPO glycoforms). The most significant single finding is that the criteria currently in use are entirely appropriate for doping control since no false positives have been found for either method in the hundreds of samples analysed. However preliminary analysis of the CIRMS data has shown that it may be desirable to modify the criteria used to enhance the detection of endogenous anabolic steroids depending on the athletes natural levels. This would be done without compromising the selectivity of the method.

It is anticipated that all goals of the first year of the project will be met on time and within budget. The program for 2004-2005 will result in the completion of part 2 of the study by September 2004 with preparation of a scientific paper on the EPO glycoform study. The second phase of the CIR measurements will begin with analysis of the steroidal diols. During the year a paper will be prepared presenting the data obtained from the completed keto steroid phase of the project.

BACKGROUND

It was recognised in the early stages of the development of an indirect test for detecting recombinant human EPO that it was essential to establish the range of variability expected for all the relevant parameters in a large group of elite athletes. Multiple samples of blood and urine were collected from over 1100 athletes and are currently stored frozen. At the time of the collection it was recognised that the samples represented a valuable resource which could be required to validate future tests for endogenous compounds. Thus the ethics approval obtained from each subject permits the samples to be used in research on the detection of doping with EPO and endogenous steroids. The samples were collected in 12 countries with representation of all major ethnic groups. Because of repeat urine collections there are some 2000 urine samples in total. The factors related to each sample which have been recorded include gender, ethnicity, age, sport, altitude and time since exercise. The sample size and diversity is such that it should be possible to determine if there are any significant effects of gender, ethnicity, exercise, sporting discipline, altitude and biological variation.

The detection and confirmation of the presence of ingested or injected endogenous steroids using CIRMS is based on the fact that the synthetic versions of the steroids have a lower proportion of carbon 13 resulting in more negative delta ^{13}C values compared with naturally produced steroids. Typically synthetic steroids have delta values close to –30 whilst the delta value of natural circulating steroids are typically –22 to –24. The CIRMS methods used to detect doping measure the delta values of steroid metabolites and compares them to the values found for precursors. The premise on which the method is based is that in a normal individual the delta values of the precursors and the metabolites will be similar. Actual measurement of these values in a wide range of subjects will provide the sound statistical basis on which to base the criteria used for determining a positive doping case.

The current method used to confirm doping with human recombinant EPO is based on the fact that recombinant EPO is significantly less acidic than urinary EPO despite the recombinant product being produced from mammalian cells containing the human gene. This difference in acidity arises from the fact that the recombinant product is less glycosylated and has fewer sialic acid residues than normal urinary EPO. In the normal human body EPO is produced in the kidney in response to low levels of oxygen in the blood. The glycosylation of the EPO protein is needed to allow it to circulate to the bone marrow where it stimulates the production of new red blood cells. The EPO protein is rapidly destroyed and is not effective if injected in the deglycosylated form. The protective effect of the sugar moieties has been extended in the new Amgen product Aranesp, which has the EPO protein modified so that two additional sugar chains are introduced. This has the effect of significantly increasing the half life of the circulating material (Egrie and Browne 2001). Although much is known of the structure of the isoforms of recombinant EPO from mass spectral and other measurements

(Ohta et al 2001) what data there is on the structure of natural EPO comes from a very few subjects. In fact virtually all the data available on the variability of natural EPO isoforms has come from the few IOC laboratories that are currently routinely performing the EPO urine test. Since the urinary EPO test is critically dependent on the difference in glycosylation between natural and recombinant EPO, it is essential to be aware of the extent of natural variability of this glycosylation in the general population of elite athletes.

AIMS

The aims of the project are twofold. Using urine samples already collected from a large cohort of ethnically and geographically diverse elite athletes we intend to:

A. Determine the variability in the isotope ratio of some selected endogenous steroid metaboliites and evaluate whether the delta ^{13}C values are significantly affected by factors such as gender, ethnicity and geographical location.

B. Determine the variability of the natural isoform pattern of urinary EPO and evaluate whether the patterns are significantly affected by factors such as gender, ethnicity and altitude.

As there are essentially two separate projects within the one grant application the results for each have been reported separately.

Part 1 CIR measurements

Experimental Methods

A method used has been developed for the extraction of the endogenous steroids androsterone (A), etiocholanolone (Et), 11-ketoetiocholanolone (11-keto), and their measurement using CIRMS. After hydrolysis with b-glucuronidase the free steroids are extracted from the urine using BondElut Certify SPE columns. The extracts are analysed by GC-CIRMS using a Finnigan-MAT Delta Plus with a Hewlett-Packard 6890 GC. The calculations of isotopic ratio are made using ISODAT 7.4 software. The $\delta^{13}C$ values for the steroid metabolites A and Et, and the precursor 11-keto have been measured. The results are stored in Excel spreadsheets prior to statistical analysis.

Results

The results from the first four countries completed Australia, China, Kenya, and New Zealand comprising some 450 samples have been published (Cawley et al 2004). In summary the major findings are:

- The GC-CIRMS methodology has a high degree of precision and accuracy based on multiple measurements of the internal standard 17-MeT. The certified value determined by combustion analysis was -32.80‰, whilst the mean value found was -32.42‰ (n>700), with a SD of 0.90.
- The distribution of $\delta^{13}C$ values was close to normal in all four countries.
- The $\delta^{13}C$ values and the spread of these values were similar for the Australian and New Zealand populations. This was to be expected as the dietary patterns in the two countries are similar.
- The $\delta^{13}C$ values were significantly different ($p<0.0001$) for China (mean = -21.6), Kenya (mean = -20.3) and Australia/New Zealand (mean = -22.8). It is almost certain that the different dietary patterns are responsible for these differences.
- There was a consistent difference of approximately 2.0‰ between the average $\delta^{13}C$ values of androsterone and etiocholanolone and the value for the precursor 11-ketoetiocholanolone.
- The most negative $\delta^{13}C$ value found in any sample for androsterone was -25.1‰ and for etiocholanolone it was -25.8‰.

Implications for Doping Control

These preliminary results have a number significant implications for the use of GC-CIRMS in the detection of the abuse of endogenous anabolic steroids:-

- The precision and accuracy of the multiple measurements adds confidence to the reliability and reproducibility of GC-CIRMS measurements.

Our laboratory like many others use a combination of criteria to assess whether a sample is positive. Our current criteria are –

1. The difference between the average of δ^{13}C A and δ^{13}C Et values, and δ^{13}C 11-keto must be greater than 4.0‰.
2. The ratio must be greater than 1.15.
3. δ^{13}C A and δ^{13}C Et must be more negative than -27.0‰.

All must be met for a sample to be called positive. As expected none of the samples measured so far would be identified as positive.

- The results show that the use of a difference of greater than 4.0‰ is a necessary condition to confirm doping but it is not a sufficient condition. A small number of samples had differences greater than 4.0‰ usually due to isotope enriched (less negative) values for the 11-keto. Thus far a difference of 6.0‰ would be a sufficient condition by itself to confirm doping.
- The use of the ratio of the average of A and Et values to that of the 11-keto provides no additional information and should be discontinued.
- The suggestion has been made that a cutoff value such as -27.0‰ could be used to detect the use of synthetic endogenous steroids (Aguilera et al 2000). This is supported both by the lowest value found being -25.8‰ and by the mean and standard deviation results. The lowest calculated δ^{13}C value (mean minus 3σ) is -26.5‰ for New Zealand. The δ^{13}C values for Australia, China and Kenya were all less negative.
- The combined criteria of a difference of 4.0‰.and δ^{13}C A and δ^{13}C Et being more negative than -27.0‰ is appropriate for the Australia/New Zealand populations but will result in a greater number of false negatives when applied to athletes with less negative δ^{13}C values such as those from Kenya.
- Although a full statistical analysis has yet to be carried out it is clear from the data so far that the effect of geography is far greater than any other effect including sex and sport. From the nature of human metabolism it would be expected that diet would be the major influence on steroid isotope ratios (Morrison et al 2000).

Part 2 EPO glycoforms

Experimental Methods

The urinary EPO concentrations have been measured using an EPO kit on a DPC Immulite instrument. As this method is designed for serum samples it was necessary to centrifuge each urine sample, ultrafilter it and buffer wash the retentate prior to analysis.
The urinary isoforms were measured using a variation of the original Lasne method (Lasne et al 2002). 20mL of each urine was concentrated to 30uL using two ultrafiltration devices. The concentrated samples underwent isoelectric focussing on an ampholyte gel and were then transferred with two Western blotting steps. The membrane was visualised using Pierce SuperSignal West Femto Maximum Sensitivity Substrate. The signals were recorded with a Fuji LAS-1000 camera and quantitated with Fuji Image Guage v3.41 software. Sample results are stored in an Access database.

Results

In order to properly evaluate the isoform data collected from elite athletes it was first necessary to determine what variability there was in the method itself and what variability was to expected in any one subject over time. The method variability was estimated by analysing urine samples spiked with varying levels of recombinant EPO from 0 to 15 IU/L several times on six gels run on different days. The results showed that a variation of 5 in a value of 50% basic isoforms could be expected (CV = 10%). At higher values (90% basic isoforms) the variation was less being about 3 (CV = 3%). The subject variability was determined by analysing the results from some 20 subjects who had up to 10 urine samples collected over a period of three days. The mean CV observed for the subjects was 17% which is much higher than the variation that could be attributed to the method of measurement. A large variation in urinary EPO concentrations was also observed in these subjects but there was no correlation observed between urinary EPO concentration and % basic isoforms.

Some preliminary results from the EPO2000 samples and the low risk ASDA samples have been evaluated. The table below summarises the results

	EPO2000		**ASDA**	
% Basic Isoforms	Male	Female	Male	Female
Mean	35.8	39.7	36.5	43.9
Median	35.5	37.0	35.0	44.0
Standard deviation	14.5	16.5	13.8	15.8
Count	100	50	163	47

The results show that the ASDA low risk routine samples and the EPO2000 samples are not significantly different in the distribution of their EPO % basic isoforms. This demonstrates the validity of including the ASDA samples in our study. From the current data there appear to be no significant effects of ethnicity on the isoform results

% Basic Isoforms	ASDA males	Chinese males	Kenyan males
Mean	36.5	37.2	40.3
Median	35	36	39
Standard deviation	13.8	17.1	11.2
Count	163	38	16

Implications for Doping Control

These preliminary results have a number significant implications for the use of urinary EPO isoforms in the detection of the abuse of recombinant EPO:-

- The reproducibility of the % basic isoform measurement using the existing amphoteric gel technique with double blotting is adequate for doping control purposes. This was determined under the worst case scenario ie. different gels prepared on different days whereas a positive sample would be measured against standards run on the same gel.
- The standard deviation of the % basic isoforms found for any given individual is approximately 17. This high individual variability means that the detection of differences relating to sport, age or sex is unlikely.
- There have been no differences observed relating to ethnicity.
- The last two findings indicate that the test should be equally applicable to all those tested.
- No positive findings have been found in the samples which indicates that the criteria used to determine doping with recombinant EPO are appropriate. Closer examination of those samples with relatively high % basic isoforms may lead to improved criteria.

Report on Progress

Part 1 CIR measurements

Summary Table

Activity	Estimated completion in proposal	Progress thus far	On schedule Y/N
Organise logistics	July 2003	Staff were allocated to the project in July 2003	Y
Organise samples	August 2003	Samples selected from existing sample set and sorted by country of origin and volume of sample available.[1]	Y
Analyse samples	June 2004	Approximately 900 samples have been extracted for GC-CIRMS analysis. 750 have been analysed.	Y
Evaluation of data	Nov/ Dec 2003 and April to June 2004	The data evaluation needed for the results entry into the database has been completed for some 700 samples from 12 countries. The statistical evaluation will begin in April 2004.	Y
Report to ADRP	January 2004	Report requested in November 2003. Preliminary report submitted in December. Second report requested in March 2004.	Y

1. The original proposal called for the analysis of most of the 2000 urine samples collected in the EPO2000 project. As there were only a little over 1100 subjects in the study many of the urine samples were duplicate collections made a week or so apart. Close examination of the samples collected has shown that not all are suitable for analysis. In addition it has been found that steroid excretion is normally stable for an individual over extended periods of time and repeat analyses from the same subject will provide virtually no additional information. As a result of these factors the number of urine samples suitable for analysis by GC-CIRMS is approximately 1050.

Part 2 EPO glycoforms

Summary Table

Activity	Estimated completion in proposal	Progress thus far	On schedule Y/N
Organise logistics	July 2003	Staff were allocated to the project in July 2003	Y
Organise samples	August 2003	Samples selected from existing sample set and criteria established for selection of further samples.[1]	Y
Analyse samples	June 2004	Urinary EPO concentrations have been measured for over 1100 EPO2000 samples. Only one quarter of the urines had sufficient EPO present to continue with isoform analysis. The data set has been extended by using samples from the operational sample base. Over 600 of these have been screened for urinary EPO concentration and some 300 have had isoform distributions measured.	Y
Evaluation of data	January and July 2004	Isoform measurements have been made but statistical evaluation is currently behind schedule.	N
Report to ADRP	January 2004	Report requested in November 2003. Preliminary report submitted in December. Second report requested in March 2004.	Y

1. In the original project proposal it was planned to use the EPO2000 urine samples to obtain the data required. However only approximately one quarter of the samples had sufficient urinary EPO present to proceed with EPO isoform analysis. This meant that there would be significantly less data available than expected. It is possible that these samples, despite being stored frozen, have undergone some degradation. Since the project was planned there has been evidence coming from several laboratories that some urine samples can change with time and produce isoform patterns that resemble recombinant EPO (Howe 2003). With fresh urines 40 to 50% have sufficient EPO to proceed with isoform analysis. For these reasons it was decided to continue the project using samples taken from the routine operational collections. Samples which have been reported negative are selected from a range of sports including some at risk endurance sports but avoiding samples where there is any indication at all from blood parameters of EPO abuse. In addition fresh urine samples have been obtained from elite athletes in Indonesia to extend the ethnic diversity and it is planned to obtain urine samples from at least one other country.

Proposed activities to June 2004

For the period up to June 30 it is anticipated that the project will continue in accordance with the schedule presented in the original project proposal. Both projects are on schedule or ahead of schedule for experimental activities. The specific activities proposed for the next months are:

- **Part 1 CIR measurements.** Extractions and GC-CIRMS analyses will continue with the intention of having approximately 1050 samples from 12 countries analysed by June 2004. Data processing and review will continue so that the statistical analysis can begin. The method for the diol analysis procedure will be finalised and validated in preparation for the second stage of the project. It appears at this stage that the method will be considerably more complex than first envisaged and the number of urine samples to be analysed will need to be reduced.

- **Part 2 EPO glycoforms** Analysis will continue so that 600 urine samples will have been tested using the gel electrophoresis method. It is anticipated that at least 25% of the samples will be from subjects having non-Caucasian ethnicity. The statistical analysis of the data will commence before June 2004 but will not be completed until September as indicated in the original proposal.